POLYSACCHARIDES FROM ALOE BARBADENSIS REDUCE THE SEVERITY OF BACTERIAL SPOT AND ACTIVATE DISEASE-RELATED PROTEINS IN TOMATO


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SUMMARY

Bacterial spot is an important disease of tomato that causes a reduction of the leaf area and affects plant yield. The objective of this study was to evaluate the effect of polysaccharides from aloe vera (Aloe barbadensis Miller) parenchyma (PAV) on the severity of bacterial spot and to detect the defense mechanisms activated by them. Tomato plants at the five true leaf stage were treated with PAV, and inoculated with Xanthomonas gardneri four days later. The effect of heating PAV suspension on its effectiveness for disease control was also evaluated. The ability of the polysaccharides to induce the synthesis of pathogenesis-related proteins in plants was determined spectrophotometrically. Finally, the antimicrobial effect of PAV on X. gardneri cultures was checked. PAV heated at 100°C for 30 min reduced bacterial spot severity by up to 85.1%, but control level was decreased when the PAV heating time was doubled. Concerning peroxidase, polyphenoloxidase, and glucanase activities in tomato plants treated with PAV, a significant increase of these enzymes was found, even before challenging plants with X. gardneri. Although PAV showed little direct effect on the bacteria it seems to constitute a good alternative for the control of bacterial spot of tomato through induced resistance.

Key words: Aloe vera, alternative control, resistance induction, Xanthomonas gardneri, bacterial spot of tomato.

INTRODUCTION

Tomato is a plant native to South America, more specifically in the region between Ecuador and northern Chile from the coast to certain altitudes of the Andes. It grows under a variety of climates and is affected by various diseases that reduce the quantity and quality of the yield (Alvarenga, 2004).

Bacterial spot, a disease caused by different species of Xanthomonas, has a great impact on tomato crops in Brazil, where it is favoured by temperatures from 20 to 30°C, being more severe in places characterized by strong winds and heavy rainfalls (Lopes and Ávila, 2005). In central-western Brazil, Xanthomonas gardneri has often been isolated from plants with this disease (Quezado-Duval and Lopes, 2005). Although healthy seeds and resistant varieties are the most effective measures for controlling bacterial spot, cupric fungicides are widely employed with the consequent risks of resistance development, inadequate control and adverse effects on the environment (Kurozawa and Pavan, 2005).

According to Campanhola and Bettiol (2003), the demand of consumers for food free from toxic residues, i.e for healthier products, have fostered the search for alternative methods for combating plant pathogens such as biological control and resistance induction, which involves the activation of latent defense mechanisms of plants through elicitor molecules. This activation is demonstrated by an increase in the synthesis of defense compounds, such as pathogenesis-related proteins (glucanases, peroxidases, polyphenoloxidases, etc.) (Heil and Bostock, 2002).

Aloe vera (Aloe barbadensis Mill.) is a medicinal plant with antimicrobial potential (Pandey and Mishra, 2009). It also contains elicitors, as shown by Jasso de Rodrígues et al. (2005) who observed the inhibition of in vitro growth of Rhizoctonia solani, Fusarium oxysporum, and Colletotrichum coccodes using liquid fractions and by Stangarlin et al. (1999) who reported that plant extracts were effective in inducing phytoalexin 3-deoxianthocyanidin in mesocotyls of sorghum.

The leaves of aloe vera have a mucilaginous parenchyma containing a great amount of polysaccharides (Carporno et al., 2009), that represent an attractive alternative to conventional plant disease control, because of their abundance and origin from renewable sources (Vianna Filho, 2009). Many of these compounds have proven effective as resistance inducers, such as chitosan, which reduced the severity of bean anthracnose (Di Piero and Garda, 2008), and oligogalacturonides derived from the cell wall of citrus, which raised the synthesis of protease inhibitors in tomato.
leaves (Farmer et al., 1991).

Thus, the objective of this study was to evaluate the effect of polysaccharides from aloe vera parenchyma against bacterial spot of tomato and to identify the biochemical mechanisms of resistance activated by them.

**MATERIALS AND METHODS**

**Cultivation conditions and plant material.** The experiments were conducted in a greenhouse and in the Laboratório de Fitopatologia of the Centro de Ciências Agrárias at Universidade Federal de Santa Catarina, Florianópolis (Brazil). Seeds of tomato cv. Santa Cruz Kada, susceptible to X. gardneri, were sown in 128-cell polystyrene trays with Germina Plant substrate (FLORESTAL, Brazil). Two weeks after sowing, seedlings were transplanted into 2 litre pots, containing soil and organic compost (5:1 v/v), and kept under greenhouse conditions (average temperature 25°C and average photoperiod 12 h through the duration of the experiment).

**Isolate of X. gardneri used in the experiment.** Researchers from Sakata Seed Sudamerica (Brazil) isolated a bacterium (Xan 166) from infected tomato leaves (cv. Carmen) collected in Aguas Mornas (Santa Catarina, Brazil) which was identified as X. gardneri (Group D) by the Centro Nacional de Pesquisa de Hortaliças (CNPH) Embrapa, Brasília. Species identification was confirmed by BOX-PCR using the primer 5′- CTACG-GCAAGGGGACGCTGACG-3′ that amplifies repetitive and conserved sequence dispersed throughout the bacterial genome. Therefore, the genomic profile of this Xanthomonas strain was compared with that of reference isolates of Xanthomonas species associated with bacterial spot of tomato: IBSBF 2363 (X. euvesicatoria), IBSBF 2364 (X. vesicatoria), IBSBF 2370 (X. perforans) e IBSBF 2373 (X. gardneri), from the Phytobacteria Culture Collection of the Instituto Biológico, Campinas (Brazil). Xan 166 was maintained in phosphate buffer (8.6 mmol l⁻¹ K₂HPO₄; 7.4 mmol l⁻¹ KH₂PO₄) at 25°C in the Laboratório de Fitopatologia at the Universidade Federal de Santa Catarina, Brazil (Coqueiro and Di Piero, 2011).

**Extraction of polysaccharides from aloe vera parenchyma.** Polysaccharides were extracted from parenchyma of adult external leaves of aloe vera, collected in March 2010 in the farm Caezel, which belongs to the company Naturama Sucos Integrais do Brasil (Paulo Lopes, Santa Catarina). Leaves were washed with running water and parenchyma was removed, crushed, and filtered. Six volumes of ethanol 92°GL were then added to a volume of the resulting gel, and the final mixture was maintained at 4°C for 24 h to allow flocculation of high molecular weight polysaccharides (PAV) which were vacuum filtered, dried in an oven at 60°C and weighed every 3 h until a constant weight was reached. Dried polysaccharides were stored at -20°C.

Before their application onto tomato plants, the deep frozen material was powdered in liquid nitrogen, dissolved in 0.05 N HCl, according to Ben-Shalom et al. (2003), and the pH of the resulting suspension was adjusted to 5.6 by adding 2 M NaOH. PAV suspensions (different concentrations) were preheated at 100°C for 30 min, cooled, and applied to tomato plants.

**Treatment of the plants, inoculation, and disease evaluation.** To verify the effect of different concentrations of PAV on the severity of bacterial spot, tomato plants were treated with hydrochloric acid (HCl) 0.05 N (pH 5.6) as a negative control, PAV was administered at four concentrations (0.75, 1.5, 3.0 and 6.0 mg ml⁻¹) and the commercially available resistance inducer, acibenzolar-S-methyl (ASM) at 25 µg ml⁻¹, served as positive control. Seedlings at the five true leaf stage were hand-sprayed each with 8 ml of every solution. Four days after the application, plants were inoculated with X. gardneri strain Xan 166 (OD₆₀₀ = 0.9) by spraying the bacterial suspension (15 ml/plant) on the abaxial and adaxial surfaces of the leaves with a subsequent incubation of the plants in a chamber for 48 h at 27±2°C, 95% RH and photoperiod of 12 h. Fourteen days post inoculation, the disease severity (percentage of damaged leaf area) was assessed on three leaves of the intermediate section of each plant using a diagrammatic scale (Mello et al., 1997).

A subsequent experiment was conducted to verify the effect of heating PAV in different combinations of temperature and time on their efficiency against bacterial spot. Thus, polysaccharide suspensions at 1.5 mg ml⁻¹ were heated at 50 or 100°C for 30 or 60 min, cooled to room temperature, and applied to the plants. HCl 0.05 N (pH 5.6) and suspension of PAV without heating (1.5 mg ml⁻¹) were used as controls. Plants were inoculated and evaluated as described above. All the experiments involving the evaluation of bacterial spot control by PAV were carried out in a completely randomized design with five replicates, and the experimental plot was represented by a pot with two plants.

**Determination of peroxidase, polyphenoloxidase, and β-1,3 glucanase activity.** For biochemical analysis, different solutions were applied to tomato plants at four true leaf stage: 0.05N HCl (pH 5.6); PAV (1.5 mg ml⁻¹), heated at 100°C for 30 min; ASM at 25 µg ml⁻¹. After 4 days, the plants were inoculated with a X. gardneri suspension, as in previous experiments. Samples for evaluation consisted of five leaflets collected from the third leaf of the plants. Sampling times were 0, 2, 4, and 6 days after spraying. The experiment was carried out in a
completely randomized design with six replicates, and the experimental plot was represented by a pot with two plants.

After collection, the samples were stored in aluminum foil and placed at -20°C. Leaf tissues, powdered in liquid nitrogen, were additioned with 1.5 ml phosphate buffer at 100 mmol l⁻¹ (pH 7.0) (containing ethylenediamine tetra-acetic acid 1 mmol l⁻¹, 1% polyvinylpyrrolidone and phenylmethylsulfonyl fluoride 1 mmol l⁻¹). The resulting slurry was centrifuged at 20,000 g for 30 min at 4°C, the supernatant (protein extract) was recovered and subjected to analysis for detecting peroxidase, polyphenoloxidase, and glucanase activities.

Peroxidase activity was determined according to Hammerschmidt et al. (1982), with adaptations. For the reaction, 50 µl of the protein extract was additioned to 2.95 ml of phosphate buffer 50 mmol l⁻¹ (pH 6.0) containing 20.2 mmol l⁻¹ guaiacol and 90 mmol l⁻¹ hydrogen peroxide. The activity of the enzyme was measured by a spectrophotometer, which measured the conversion of guaiacol into tetraguaiacol. The reaction was conducted for 4 min at 40°C recording the optical density values every 30 sec. Results were expressed in units of optical density at 470 nm per mg protein per min (OD₄₇₀ nm mg protein⁻¹ min⁻¹).

Polyphenoloxidase activity was determined using a method adapted from Duangmal and Apentem (1999). For the reaction, 200 µl of protein extract was additioned to 2.8 ml of phosphate buffer 100 mmol l⁻¹ (pH 7.0) containing catechol 60 mmol l⁻¹. The enzyme activity was determined spectrophotometrically measuring the oxidation of catechol converted into quinone. The reaction was conducted for 1 min at 40°C by recording the values of optical density at every 3 sec. The results were expressed in units of optical density at 420 nm per mg protein per min (OD₄₂₀ nm mg protein⁻¹ min⁻¹).

Colorimetric quantification of reducing sugars released from laminarin was used to determine the glucanase activity. For the reaction, 50 µl of protein extract in 450 µl of laminarin (750 µg ml⁻¹) dissolved in sodium acetate buffer 0.1 M (pH 5.0) was used. After incubation at 44°C for 1 h, 50 µl of the mixture were diluted with 450 µl of distilled water and additioned with 1.5 ml of 1% hydrazide of p-hydroxybenzoic acid (Lever, 1972). The final solution was incubated at 100°C for 5 min and read by a spectrophotometer at 410 nm. Glucanase activity was expressed in µKatal per milligram of protein in which 1 Katal is defined as the enzymatic activity that catalyzes the formation of 1 mole of glucose-equivalent per second (Di Piero and Pascholati, 2004). Total protein content was determined according to Bradford (1976) using Coomassie brilliant blue, and bovine serum albumin as standard protein.

Antimicrobial activity of PAV. Aliquots (100 µl) of PAV suspension (0.75, 1.5 and 3.0 mg ml⁻¹) or HCl 0.05 N (pH 5.6) were spread on the surface of sterile Petri dishes containing nutrient agar (NA) [meat peptone 5.0 g, meat extract 3.0 g, agar 12 g in 1 litre distilled water (Merck, Germany)] previously autoclaved at 121°C for 20 min, with the aid of Drigalsky stick. After 1 h, 100 µl of a 10⁷ CFU ml⁻¹ suspension in sterile distilled water (pH 7) of a 48 h NA culture of X. gardneri, Xan 166 (corresponding with an optical density of 0.3 absorbance units at 600 nm) was spread on NA plates prior to incubation at 25°C for 48 h. A complete random experimental design was used with six replicates. The bacterial growth was evaluated by preparing, from colonies typical of X. gardneri, a cell suspension in 5 ml distilled water and the absorbance was monitored at 600 nm.

Statistical analyses. Analysis of variance and Tukey’s test at 5% probability were used to analyze the difference among the means of the variables. Analyses were performed using the statistical program Statistica 8.0 (Statsoft, 2007). The experiments involving the effect of PAV concentrations were assessed through regression analysis using the statistical software Sisvar (Ferreira, 2003).

RESULTS

All PAV concentrations tested reduced the severity of bacterial spot in tomato plants when applied 4 days before inoculation, similarly to the effect provided by the systemic resistance-inducer ASM. PAV concentrations of 0.75 and 1.5 mg ml⁻¹ reduced disease severity (DS) by 71.5%, and concentrations of 3.0 and 6.0 mg ml⁻¹ reduced it by 85.1% (Fig. 1). Statistical differences in DS among treatments were also observed in the trials where

![Fig. 1. Effect of concentrations of polysaccharides from aloe vera parenchyma (PAV) on the severity of bacterial spot of tomato caused by Xanthomonas gardneri. Average severity of plants treated with acibenzolar-S-methyl: 1.2%. * Significant at 5% probability.](image-url)
PAV was heated before use as a resistance inducer. The lowest DS values were found in plants treated with PAV previously heated at 50°C for 60 min or at 100°C for 30 min, which induced an average reduction of 51.5% compared to the control (Fig. 2). DS in plants treated with polysaccharides heated at 50°C for 30 min and with non-heated PAV suspension was in average 36.5% lower than in the control plants. However, tomato plants treated with PAV heated at 100°C for 60 min showed a DS only 15% lower than the control (0.05 N HCl) (Fig. 2).

As to peroxidases and polyphenoloxidases, it was found that tomato plants treated with PAV (1.5 mg ml\(^{-1}\), heated at 100°C for 30 min) showed enzyme activity levels 5 and 1.5 times higher than those detected in plants treated with HCl 0.05 N, before inoculation with X. gardneri (Fig. 3 and 4). At the time of inoculation, i.e., four days after spraying the suspensions, both enzymes presented levels eight times higher than those of the control, demonstrating that enzyme activity was influenced by PAV application. A significant difference was found between treatments, as early as 2 days after application, when the glucanase activity was assessed (Fig. 5). In aver-

![Fig. 2. Effect of temperature and heating time of polysaccharides from aloe vera parenchyma (1.5 mg ml\(^{-1}\)) on the severity of leaf spot of tomato caused by Xanthomonas gardneri.](image)

![Fig. 3. Peroxidase activity in tomato leaves treated with polysaccharides from aloe vera parenchyma (PAV) at 1.5 mg ml\(^{-1}\) (△), acibenzolar-S-methyl (ASM) at 25 µg ml\(^{-1}\) (●) or 0.05 N HCl (■) and subsequently inoculated with Xanthomonas gardneri (Xg). The arrow indicates the moment of bacterial inoculation. The bars of errors indicate the standard deviation. The same letters in the same sampling time do not differ from each other by Tukey’s test (p <0.05).](image)

![Fig. 4. Polyphenoloxidase activity in tomato leaves treated with polysaccharides from aloe vera parenchyma (PAV) at 1.5 mg ml\(^{-1}\) (△), acibenzolar-S-methyl (ASM) at 25 µg ml\(^{-1}\) (●) or as control 0.05 N HCl (■) and subsequently inoculated with Xanthomonas gardneri (Xg). The arrow indicates the moment of bacterial inoculation. The bars of error indicate the standard deviation. The same letters in the same sampling time do not differ from each other by Tukey’s test (p <0.05).](image)

![Fig. 5. Glucanase activity in tomato leaves treated with polysaccharides from aloe vera parenchyma (PAV) at 1.5 mg ml\(^{-1}\) (△), acibenzolar-S-methyl (ASM) at 25 µg ml\(^{-1}\) (●) or 0.05 N HCl (■) and subsequently inoculated with Xanthomonas gardneri (Xg). The arrow indicates the moment of bacterial inoculation. The bars of errors indicate the standard deviation. The same letters in the same sampling time do not differ among each other by Tukey’s test (p <0.05).](image)
age, this activity in plants treated with PAV (1.5 mg ml⁻¹) was 1.4 times higher than in the control.

Plants previously treated with ASM (25 µg ml⁻¹) showed an increase in the peroxidase, polyphenoloxidase, and glucanase activities from two days after application (Fig. 3, 4, and 5). However, the increase in peroxidase and polyphenoloxidase activities was significant only after challenging plants with the pathogen (Fig. 3 and 4). On the other hand, glucanase activity in plants treated with ASM increased only slightly after inoculation with X. gardneri (Fig. 5).

Concerning the antimicrobial action of PAV on X. gardneri, it was found that the highest PAV concentration (3.0 mg ml⁻¹) reduced bacterial growth by 31%, and that the lowest concentrations (0.75 mg ml⁻¹ and 1.5 mg ml⁻¹) only by 14% as compared to the control (Fig. 6).

DISCUSSION

Polysaccharides are a good alternative to conventional control of plant diseases, because, in addition to being plentiful and readily available, they are obtained from renewable sources such as algae and plant (Vianna Filho, 2009). Because of this, studies have been conducted to verify the potential of various polysaccharides for controlling plant diseases. For example, Coqueiro and Di Piero (2011) reported that chitosan reduced the DS of bacterial spot of tomato, for it exerted an antimicrobial effect on the pathogen and increased the peroxidase activity in treated plants.

In the present study, a clear inhibitory effect of PAV on the severity of bacterial spot of tomato was observed, in accordance with the notion that aloe vera may have a direct toxic effect on certain pathogens. Saks and Barkai-Golan (1995), for example, showed the antifungal activity of PAV against postharvest citrus pathogens such as Penicillium digitatum, P. expansum, Botrytis cinerea and Alternaria alternata, whereas Stadnik et al. (2003), reported that aloe vera extracts activate defense mechanisms of cucumber plants against powdery mildew. Thus, the protection afforded by PAV in tomato plants may be due to antibiosis or induced resistance.

In the first assay, PAV heated to 100°C for 30 min reduced the severity of bacterial spot at all concentrations tested. Afterwards, we found that the thermal treatment has changed the control efficiency of PAV. According to Bongiovani (2008), exposing polysaccharides to different types of acids or temperatures may lead to the release of mono- and oligosaccharides. Boller (1995) stated that oligosaccharides are prototypes of signals that are recognized by plant cells which, in turn, induce the accumulation of phytoalexins and the activation of plant defense mechanisms. In line with Boller (1995) statement, we suggest that the heating of PAV at 50°C for 60 min or at 100°C for 30 min may have broken the bonds of polysaccharide monomers, thus releasing oligosaccharides that, acting as defense response elicitors, increase the efficiency of PAV in bacterial disease control. However, this ability may have been damaged by overheating (100°C for 60 min) due to the possible destruction of oligosaccharide elicitors. These results suggest that PAV may be acting as an elicitor, since the exposure to different temperatures has influenced the efficiency of the polysaccharides in reducing bacterial spot severity.

Regarding the effect of PAV on tomato plant metabolism, a significant increase in peroxidase, polyphenoloxidase, and glucanase activities of the treated plants was found. This increase in the level of certain enzymes may have contributed to reduction of DS because these proteins create adverse conditions for the development of the pathogen in the plant, slowing disease progression (van Loon et al., 2006). For example, peroxidases are cell membrane-associated glycoproteins involved, among other processes, in the deposition of lignin in plant cell walls (Mandal et al., 2009). Polyphenoloxidases are a group of enzymes activated after elicitation of plants and responsible for catalyzing the oxidation reaction of polyphenols, turning them into quinones, which are antimicrobial molecules (Kuhn, 2007). Glucanases are hydrolases which act on the β-glucan present in cell wall of many pathogens, promoting the disorganization of this structure and cell death (Bol and Linthorst, 1990).

Elicitor-induced metabolic changes have been already reported. For instance, Di Piero and Pascholati (2004) found an increase in the activity of β-1,3-glucanase in tomato plants treated with ASM prior to inoculation with X. vesicatoria, finding the peak of enzyme activity at 6 days after treatment, with an increase in the activity after inoculation with the pathogen, in agreement with the results of this work. Campos et al. (2004) found that plants treated with C. lindemuthianum (inductive fungus) and salicylic acid showed increased en-
zyme activity and consequent reduction of DS when assessing the influence of peroxidase and polyphenoloxidase in the resistance of bean plants to anthracnose.

In addition to the induction of resistance, PAV may also have reduced DS in tomatoes by antibiotic effect. According to Waksman (1959), a substance is only considered as antimicrobial when, at low concentrations, it is able to inhibit the growth or multiplication of microorganisms. Because of the high concentrations of polysaccharide used in the trial and the low inhibition of in vitro bacterial growth, this direct action is seen as insufficient for the level of disease control achieved in the experiments with tomato plants.

Thus, it is suggested that the control of bacterial spot by applying PAV on tomato plants is related to the induction of resistance. Key observations are the reduction of DS, absence of a significant antimicrobial activity and the increase in the activity of the enzymes responsible for the biosynthesis of plant defense compounds at a critical time for the onset of bacterial spot.

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